NEW YORK PATHOLOGICAL SOCIETY

ABSTRACTS OF PAPERS AND DISCUSSION

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The Nature and Origin of Fibrinoid: Morphological, Histochemical and Histoenzymatic Studies

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Much controversy exists concerning the nature and origin of fibrinoid. The various theories derive it from collagen, ground substance, nucleoprotein or fibrin. The following observations were made on human and experimental fibrinoid:

Experimentally, fibrinoid was produced by a number of methods all causing injury to connective tissue. Mechanical injury to the skin of rabbits resulted following incision, chemical injury following injection of trypsin, and radiation injury after insertion of radon seeds or x-radiation. Allergic injury of both skin and joint was produced by eliciting the Arthus phenomenon. Lesions of systemic hypersensitivity with fibrinoid were produced with one or more massive intravenous doses of foreign protein. Fibrinoid in a number of lesions from the "diffuse collagen diseases," arteriosclerosis, placentae and peptic ulcers constituted the human material of our studies. Morphologic and histochemical studies were carried out on the fibrinoid of both the experimental and human lesions.

The experimental lesions were studied at serial time intervals. The earliest change, in all types of lesions, was an acute inflammation in which varying amounts of fibrin could be demonstrated. At all stages, fibrin with other plasma constituents had conglomerated and inspissated, thus having all the characteristics of fibrinoid. Fibrinoid was acidophilic (eosinophilic), homogeneous and refractile, and gave staining reactions similar to fibrin. It stained red with trichrome or azan, dark blue with phosphotungstic acid hematoxylin, and red-purple with PAS. With the aid of histochemical methods, the amino acids tyrosine, tryptophane, cysteine and cystine could be demonstrated in both reticular fibrin and homogeneous fibrinoid. Incubation of frozen-substituted or absolute alcohol-fixed sections with trypsin (Nutritional Biochemicals) resulted in digestion of both fibrin and fibrinoid, while collagen remained unchanged. Acid mucopolysaccharides were decreased in the earliest exudative lesions and increased as regeneration and repair commenced, and young connective tissue was laid down.[1, 2] Fibrinoid was embedded in this material. Following incubation with testicular hyaluronidase (Wyeth) the staining of acid mucopolysaccharide was inhibited or diminished, but the fibrinoid remained unchanged.

In the human lesions, all the various stages of fibrinoid formation could be traced from delicate threads of fibrin to bands, clumps, and large masses which showed all the morphologic and tinctorial characteristics of fibrinoid described above.

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Histochemically, fibrin and fibrinoid gave all the reactions found in the experimental fibrinoid. A hematoxylinophilia was at times associated with fibrinoid in disseminated lupus, but it was found occasionally also in rheumatoid arthritis and periarteritis nodosa. The fibrinoid always gave a positive Feulgen reaction. Possibly the most significant finding was the digestion of fibrinoid with fibrinolysin (Parke, Davis) in frozen-substituted or alcohol-fixed tissues. Trypsin had a similar effect. Collagenase (Burroughs, Wellcome & Co.) treatment caused loss of staining of collagen but did not affect the fibrinoid.

Acid mucopolysaccharides could be demonstrated only in advanced lesions showing fibrinoid. The reason for this is that they are associated with repair, i.e., formation of young connective tissue.[1-4]

The demonstration of increased amounts of mucopolysaccharides in association with fibrinoid has led some investigators[5] to conclude that fibrinoid is derived from a precipitation of mucopolysaccharides. However, in the earliest exudative lesions they are diminished or at least not demonstrable by histochemical methods.[1, 3]

From these studies there is ample morphologic evidence that fibrinoid derives from conglomeration and inspissation of plasma, particularly fibrin. Histochemical evidence supports this view: the amino acids which can be demonstrated histochemically in fibrinoid are present in fibrin and other plasma proteins, but are absent or in low concentration in connective tissue. In alcohol-fixed tissue, fibrinoid is digested by trypsin and fibrinolysin, whereas collagenase and hyaluronidase alter only the connective tissue, but not the fibrinoid.

It was concluded from these studies that fibrinoid derives from fibrin which is exuded during the course of a fibrinous or serofibrinous inflammation.[6, 7] There is little evidence that fibrinoid derives from collagen or connective tissue ground substance. Degenerating elements of connective tissue, i.e., collagen and ground substance, and also nuclear material and various plasma constituents, may be intimately associated with fibrin in the formation of fibrinoid, but fibrin is the only constant component. Without fibrin there can be no formation of fibrinoid, or, at least, of the substance which Neumann (the originator of the term) has defined as an acidophilic, homogeneous and refractile material with the staining characteristics of fibrin.

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DISCUSSION

LOUIS ODESSKY: I would like to know exactly when does fibrin become fibrinoid when you see it microscopically. When can you say you are looking at fibrin and when at fibrinoid?

ALFRED ANGRIST: Will this presentation necessarily rule out the active participation of fibrinogen in the formation of fibrin by heparin and other acidic polysaccharides, etc., suggested by the work of Altshuler, Thomas, and others?

SIGMUND L. WILENS: Dr. Cooper I know is interested in this subject; has he anything he would like to say?

NORMAN S. COOPER: I would like to congratulate the author on an excellently presented paper. I think there has been considerable accumulating evidence that the term "fibrinoid" is a red (or Orange G) herring and this material certainly supports the concept that it is really old fibrin.

HENRY Z. MOVAT: The first question I am afraid I cannot answer fully. We were taught in medical school to call fibrin threads and meshworks with definite staining characteristics. Fibrinoid on the other hand is defined as clumps and bands of an acidophilic and refractile material with the staining properties of fibrin.

Two years ago at Queen's University in Canada, seeing the pathology class slides showing pericarditis in a case of lupus (representing an example of fibrinoid) and one of uremic pericarditis (used as an example of fibrinous exudate), I covered up the labels and showed the slide to one of our demonstrators. He diagnosed both as fibrinous pericarditis, although he taught the students that in the case of lupus, the material was "fibrinoid". Transition between fibrin and fibrinoid occurs frequently as demonstrated in the slides which I presented.

In regard to the question by Dr. Angrist, I would like to say that the role of acid mucopolysaccharides in the formation of fibrinoid as suggested first by Altshuler and Angevine has not as yet been definitely established. Dr. Angevine seems to believe now that fibrinoid derives from some sort of plasma components, as pointed out by him some two years ago when I presented the preliminaries of this work. He still believes, however, in the precipitating role played by the mucopolysaccharides as suggested in their original work. In our studies dealing with experimental fibrinoid we could not demonstrate an increase but rather a decrease of acid mucopolysaccharides in the early exudative lesion. Tissues rich in polysaccharides showed a swelling or mucinous edema. At later stages an increase of these substances could be demonstrated in connection with early repair. I am inclined to think that depolymerization of polysaccharides is followed by an increased permeability of the ground substance, thus facilitating the exudation of plasma proteins.

SIGMUND L. WILENS: What is the purpose of retaining the term "fibrinoid"? Do you think it should be discarded, and one should just use fibrin?

HENRY Z. MOVAT: No, I do not believe it should be discarded. People will use the term just as they will continue using the term "collagen diseases" which some people tried to discard but without success. Fibrinoid is not fibrin after all; it is a physically altered fibrin.

SIGMUND L. WILENS: You think then there is sufficient reason for retaining the term?

HENRY Z. MOVAT: Yes, I do.

*Recent Advances in Enzymatic Histochemistry** *(Abstract)*

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Of the multitude of enzymes known, only relatively few hydrolytic and oxidative ones can be visualized by histochemical techniques at present. Those demonstrable are not necessarily the most important ones, but have been developed mostly because of fortuitous circumstances.

The biochemist, in studying enzymatic reactions in tissue extracts, can use all methods of modern chemistry for their recognition and measurement, as, for instance, color reactions, gas analysis, etc. The histochemist is almost exclusively limited to the use of color reactions. The biochemist destroys the tissue structure and prepares liquid extracts in which biochemical reactions can be studied under optimal conditions. The histochemist in contrast must preserve the integrity of the tissue and considers as a minimal goal the correct localization of enzymatic activity at a cellular and as a maximal on a subcellular level. He is, therefore, confronted with the problem of adequate tissue preparation for best results.

For this purpose various methods can be employed. A) Frozen sections from fresh unfixed material cut either on a conventional or a Sartorius freezing microtome[1] or in a Linderstrom-Lang type cryostat at low temperature.[2] B) Paraffin sections from frozen-dried material.[3] C) Frozen sections of tissues fixed in neutral cold formalin. D) Paraffin sections of tissue fixed in cold acetone followed by routine dehydration techniques.[4-6]

It is obvious that one of the products formed by enzymatic action must be precipitated in insoluble form at the exact place of its origin. This substance must be itself colored or be convertible into a colored product. The following procedures are at present used for this purpose. A) The conversion of products of enzymatic hydrolysis into compounds of lead, cobalt or copper which can be visualized as dark sulfides. This principle is used in procedures for demonstration of various phosphatases, choline esterase,[4-6] carbon-anhydrase,[7] etc. B) Coupling reactions in which diazonium salts will form brilliant azodyes with the products of enzymatic hydrolysis. This technique is finding an ever-increasing application and is used for the demonstration of alkaline and acid phosphate, esterases,[4-6] glucuronidase,[8] etc. C) The formation of insoluble colored products either by the action of oxidative or reducing enzymes.[4-6] Various dehydrogenases can be demonstrated by the formation of insoluble tetrazolium salts or insoluble tellurium.[9] D) The formation of an insoluble substance visualized by a specific staining reaction as in the case of phosphorylase.[10]

The question of exact localization of enzymatic activity is far from settled. Most investigators agree that in the Gomori technique for alkaline phosphatase a nuclear reaction is spurious and does not represent a true picture of enzymatic localization. Holt[11] in a thorough study has recently applied improved techniques in an attempt to establish localization of esterase on a subcellular level. He found this enzyme in discrete bodies in the cytoplasm of renal and hepatic cells in the rat. This however, does not agree with findings established by differential centrifugation, since esterase is connected with the microsome fraction and not with larger structures.[12]

Closely related to the problem of exact

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localization is the problem of choosing the correct tissue preparation. For some enzymes, for instance, succinic dehydrogenase or glucose-6-phosphatase, unfixed frozen sections or frozen-dried sections must be used since any type of fixation destroys these enzymes. Occasionally activity is differentially inactivated by fixatives. Adenosinetriphosphatase activity in striated muscle is completely abolished by formalin fixation, but staining of vascular endothelium is not diminished.[13]

In a recent study, Nachlas, Prinn and Seligman[14] have shown that large portions of some enzymes diffuse into the surrounding medium from fresh frozen sections while previous fixation of the material leads to a greater retention of enzyme activity. There are, however, great quantitative differences between various enzymes tested. Acetone fixation followed by conventional tissue preparation and paraffin embedding causes considerable loss of enzymatic activity.[15]

From the point of view of histochemical localization, quantitative differences appear less important than shifts in cellular distribution of enzymes. Does the better definition of structures often seen in formalin fixed, as compared to unfixed frozen sections, necessarily reflect the true state as it occurs in the living cell? Considerable differences in staining patterns of alkaline phosphatase can easily be detected in fresh and formalin fixed kidney sections.[16] In unfixed sections the cytoplasm stains diffusely while in fixed sections staining occurs preferentially in brushborders. In fixed sections from the rat kidney stained for phosphatase or 5-nucleotidase at pH 72, activity of the proximal convolutions is localized moreover in small bodies not unlike those described by Holt in his preparation of esterase.

The histochemical specificity of some staining reactions can be more clearly delineated by the inclusion of activators and inhibitors in the incubation mixtures. Thus cholinesterase can be separated from nonspecific esterases by its sensitivity to eserine.

From a practical point of view it is important to realize that in biopsies from human sources and in tissues from animals, enzymes are apparently more stable than is usually assumed and that histochemical distribution patterns in many instances remain unchanged for comparatively long periods of time. On the other hand, postmortem material often gives disappointing results. The influence of agonal changes must be further evaluated. Differences among various species must also be taken into account. While some reactions are limited to one or several species, others occur in certain tissues of all mammalian organs so far examined. Alkaline phosphatase and succinic dehydrogenase activity, for instance, are found without exception, in the proximal convoluted tubules of the kidney.

In this paper only a few examples of enzymatic staining reactions can be given. Succinic dehydrogenase is demonstrable in many organs.[17] The heart gives regularly a very strong reaction. The tongue shows a strong reaction closely resembling that of the heart, while the skeletal muscles show great variations in individual fibers.[18] The distribution of reduced tetrazolium particles indicating enzymatic activity in the muscle and heart resembles closely that of stainable mitochondria. In myocardial infarction, a decrease of enzymatic activity is noticed occasionally within three hours after the onset of clinical symptoms.[19] This time relation may, however, be incorrect since, in experimental infarction in the dog, diminution of activity becomes noticeable only after 12 to 15 hours.[20] Two other histochemical findings in the heart deserve mention. In the rat, the intercalated discs give a positive staining reaction when pyridoxal phosphate at pH 9[21] or muscle adenylic acid at pH 7.2 is used as substrate.[22] This is of particular interest in view of findings with the electron microscope suggesting that intercalated discs interrupt the muscle fibers and that, therefore, a myocardial syncytium does not exist.[23] The conductive system of the heart is characterized by the presence of true acetylcholinesterase. This specific enzyme is not present in the myocardium.[24]

Eserin-sensitive acetylcholinesterase can also be demonstrated in the motor endplates of striated muscle. This staining reaction can be reversibly influenced by anticholinergic drugs[25] and disappears after nerve dissection.[26]

In the kidney, a number of enzymatic reactions can be localized in various portions of the nephron.[16] It is not surprising that the most consistent staining reactions for various enzymes should be given by the proximal convoluted tubules in which most of the work load of the kidney is performed. Some enzymes are also found in the collecting tubules, for instance, DPN-diaphorase, esterase and glucuronidase. DPN-diaphorase,[27] one of the enzymes involved in the tricarboxylic acid cycle, can also be visualized in thin limbs of Henle's loops and in cells composing the glomeruli.

In experimentally induced renal necrosis, enzymatic changes occur at a faster rate following the actions of various poisons as, for instance, mercury or DL-serine, than following the ligation of the renal artery.[28] Of various enzymes tested, succinic dehydrogenase proved to be the most sensitive. After the administration of a toxic dose of mercuhydrin it disappeared within three to six hours from the proximal convoluted tubules, while even 24 hours after arterial ligation residual enzymatic activity was still present. Regenerating tubules regain their enzymes only slowly. First to reappear is succinic dehydrogenase.[16]

Not only complete necrosis, but less drastic changes influence the enzymatic staining of renal tubules. Following experimental ureteral ligation, alkaline phosphatase[29] as well as other enzymes[16] disappear from the hydronephrotic kidney. In the experimental Masugi type of nephritis, various enzymes diminish rather rapidly in renal tubules.[30]

Glucose-6-phosphatase, one of the most important enzymes involved in intermediary carbohydrate metabolism, can easily be demonstrated in liver sections.[31] Its diminution in some cases of glycogen storage disease has been reported.[32] In view of the increase of serum alkaline phosphatase in liver disease, the stainability of bile capillaries with the technique for alkaline phosphatase is of great interest. However, these structures are more regularly demonstrable when adenosine triphosphate at pH 7.2 is used as substrate.[33] In surgical biopsies of patients with obstructive jaundice and in livers of animals with experimental biliary obstruction, some bile canaliculi show marked dilatation; others show reduced enzymatic activity especially after longer lasting obstruction. Necrotic cells[34] as well as liver cells with carcinomatous transformation in ethionine-induced hepatoma show no reaction of bile canaliculi.[22]

Recently the possibility of visualizing the activity of phosphorylase in tissue sections has been reported. The effect of this enzyme can be recognized by the deposition of newly formed glycogen.[10] It can be demonstrated particularly well in striated muscle.[35]

It has been suggested that we make use of the abundance of acid phosphatase in the prostate for the identification of tumors of prostatic origin.[36, 37] In fresh or formalin-fixed frozen sections from carcinoma of the prostate a large amount of stainable acid phosphatase is demonstrated in most cases.

With the problem posed by the action of Diamox (sodium acetazoleamide) a technique for the histochemical demonstration of carbonanhydrase[7, 38] could be expected to help in evaluating the effect of this carbonanhydrase inhibitor. In our hands, good results with this method were only obtained in the rat stomach in which a remarkable staining reaction was observed in the parietal cells. This was not influenced by Diamox administration *in vivo* or *in vitro* unless enormous amounts of the drug were added to the incubation mixture.[39]

In closing, it might be of interest to refer to an observation first reported ten years ago before this Society. It could be shown that the mature leukocytes in slides from patients with chronic myeloid leukemia exhibited little or no reaction for alkaline phosphatase. In contrast, the leukocytes of patients with a myeloid reaction or with infectious leukocytosis showed a very strong reaction.[40] This observation has only recently been confirmed by various investigators[41, 42] and can be used as a practical method for the differentiation of a true myeloid leukemia from a myeloid reaction.

While much basic research will have to be done, histochemistry in general and enzymatic histochemistry in particular will undoubtedly be of increasing importance in general and experimental pathology.

DISCUSSION

ANNA GOLDFEDER: I wish to comment on Dr. Wachstein's remark that chemists are overly concerned with the freezing of tissues immediately after their removal from the animal organism for enzymatic reaction studies.

There is justification for freezing the respective tissues for metabolic investigations, not only immediately after their removal from the organism, but even for freezing them while still *in situ*. For example, phosphocreatine in the brain tissue was first detected when liquid air was poured on the brain of the living animal. This holds true for other intermediary metabolites which are very labile.

Activity of certain enzymes may proceed in excised tissues as long as the enzymes are provided with the required substrates which are preserved in the "reservoir" of cellular constituents. Dr. Wachstein's observations of enzymatic activity in tissues after they have been removed from the intact animal after some length of time may be explained on this basis.

MAX WACHSTEIN: Dr. Goldfeder is, of course, entirely correct. For many investigations involving intermediary metabolism tissues must be frozen immediately. However, those enzymes which at present can be demonstrated histochemically are considerably more resistant to higher temperature and postmortem changes than has been assumed previously.

SIGMUND L. WILENS: I think the lack of discussion is not due to the fact that we are not interested and impressed by the material you presented, Dr. Wachstein, but is due rather to the fact that few of us feel qualified to discuss the problem intelligently. I think many of us are old-fashioned tissue readers and are used to the traditional methods of examination of tissues by old-fashioned techniques. I think some of us wonder if at the present time you can give us any examples of a lesion which we might fail to recognize by the ordinary methods and which this relatively new science of enzymatic histochemistry would reveal.

MAX WACHSTEIN: As a tissue pathologist, I believe that ordinary staining techniques will almost invariably permit a satisfactory evaluation of the underlying pathology. For instance, a prostatic carcinoma can be recognized without resorting to specific staining for acid phosphatase, although occasionally this might help to identify the origin of a metastatic lesion.

Commenting on the previous presentation by Dr. Movat, one might say that fibrinoid is usually recognized by an ordinary hematoxylin and eosin stain, but that the use of histochemical methods can contribute to the understanding of the fundamental nature of abnormal tissue reactions. The application of enzymatic histochemistry to the kidney certainly must impress all those who are only accustomed to use the ordinary staining techniques. Changes which occur in the kidney under experimental conditions are very impressive. How far the application of these techniques will advance the practice of pathology is, of course, entirely undecided. In closing, may I just point out again that the use of the stain for alkaline phosphatase is very useful for the differentiation of chronic myeloid leukemia from leukemoid states.

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ABSTRACTS OF PAPERS AND DISCUSSION

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How Specific Should a Tumor Antigen Be?

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Studies of the distribution of antigenic constituents of neoplastic tissues using heterologous antibody as a specific reagent must be clearly distinguished from studies of host response to such constituents. Analysis of tissues with antibody reagents is being pursued by many groups using such diverse methods as conventional *in vitro* precipitation and complement-fixation; some newer modifications such as gel diffusion and staining with fluorescent antibody; and provocation of anaphylaxis in guinea pigs. These analytical methods are not capable of *proving* that an antigen is "specific", that is, unique for a certain tissue. What they are able to show is whether a difference exists between the quantity of some constituent in one tissue and the quantity in other tissues. When these differences are sufficiently large, they are exploitable in several ways. To make use of these differences, we must be able to determine them with both precision and accuracy. For this reason, *in vivo* methods such as anaphylaxis, or histologic methods using labelled antibody, suffer from much greater limitations than *in vitro* techniques.

The question "How Specific Should a Tumor Antigen Be?" attempts to focus attention on the fact that "specific" cannot be defined by analysis. It is equivalent to asking "How large should a quantitative difference be before we call it qualitative?" Since this question has no answer, the proper yardstick by which to judge analytical studies of tumor antigens is "How much of a difference do you find, and with what precision and accuracy can you determine it?" The problem of absolute specificity can be resolved only by isolation and characterization of the substances which react with the antibody reagent.

DISCUSSION

SIGMUND L. WILENS: Dr. Rapport has asked a provocative question and provided some provocative answers. I wonder if there is anyone in the audience who would like to discuss the matter, or ask any questions. Since we are merely pathologists, not capable of evaluating some of the material which Dr. Rapport has presented to us, I have asked Dr. Chandler Stetson to be our discussor.

CHANDLER A. STETSON, Jr.: I was delighted when Dr. Wilens asked me to discuss this paper. Both the title and the presentation were fascinating, and I think this is the first time I have heard this question raised in just this provocative and detailed fashion. I would like to discuss it from the point of view of an interested, non-expert bystander.

I think Dr. Rapport did well by defining terms, or perhaps by suggesting the redefining of some of our terms. As long as the science of immunology dealt primarily with bacteria and bacterial antigens, we were inclined to think in a rather circumscribed way of what "antigen" or "antibody" should properly mean. Perhaps now the term "antigen" should, as Dr. Rapport suggests, be re-defined as "something which will react with an antibody". A critic of this point of view would certainly insist on drawing a line somewhere, and excluding non-specific chemical reactants, but I suppose that would be quibbling.

I think that Dr. Rapport's general line of investigation aimed toward diagnosis of tumors is an extremely intriguing and I think promising one. It has been intriguing to people for a long time, of course, but I think perhaps it does hold more promise now than it did 20 years ago, partly because our ideas about specificity are becoming more sophisticated, as Dr. Rapport pointed out. His notion that there is perhaps no such thing as absolute qualitative specificity in an antigen-antibody system is exciting, and I wonder whether in the future there may be two kinds of immunology, one in which there is qualitative specificity, and one in which it is only quantitative, analogous to the heterograft-homograft system.

The other main objective of this sort of investigation on tumor antigens, as Dr. Rapport mentioned, is to get some notion of new antigens, or antigens in detectable quantities in tumor cells not present in normal cells, and this sort of information may give us some insight into the mechanism of oncogenesis. It seems to me this is at least as important as the other line of investigation, and as Dr. Rapport indicated, has already provided some fascinating leads and clues.

Abnormal Antigens in Multiple Myeloma (Abstract)

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Most patients who have multiple myeloma synthesize large amounts of electrophoretically homogeneous serum proteins. These proteins may have electrophoretic mobilities ranging from that of very slow gamma globulin to that of alpha-2 globulin. The urines of approximately 50 per cent of these patients will contain Bence Jones proteins, i.e., proteins of low molecular weight which precipitate in acid medium at 60° C and redissolve on boiling. For the last few years we have studied the antigenic properties of both these serum and urinary proteins and compared them with normal gamma globulins. This study was facilitated by the introduction of the Ouchterlony technique[1] in which antigens and antibodies diffuse towards each other through an agar medium. Lines of specific precipitate form in the region of optimal proportions for each antigen-antibody system. When three reservoirs are arranged in a triangle and the same antigen is placed in two and antibody in the third, the lines coalesce. If one reservoir contains the immunizing antigen and the other a closely related cross-reacting antigen, a spur forms at the site of fusion of the lines.[2] In our work, antisera were prepared against normal human gamma globulin and multiple myeloma proteins of three patients. The anti-gamma globulin sera were exhaustively absorbed with normal human serum albumin and, in later studies, also with a beta globulin fraction. These absorbed antisera formed one line only with normal gamma-2 globulin and cross-reacted with all purified multiple myeloma serum proteins regardless of their electrophoretic mobility, which ranged from gamma-2 globulin to alpha-2 globulin. The spurs that are formed between the lines of the gamma globulin and the mul-

tiple myeloma proteins indicate that the latter lack some antigenic groupings of normal gamma globulin.[2, 3] We have fractionated normal gamma globulin by various methods into components with different electrophoretic mobilities, but were unable to differentiate these components from the unfractionated gamma globulin. Multiple myeloma proteins from different patients are antigenically dissimilar, and we have shown that they can be placed in three main antigenic groups.[3] There is no correlation between antigenic grouping and electrophoretic mobility. A study comprising over 100 multiple myeloma sera shows that approximately 45 per cent belong to group I, which is antigenically closely related to normal gamma globulin; 35 per cent belong to group III, which is antigenically most abnormal, and 20 per cent belong to group II. All beta globulins and one alpha-2 globulin belong to group III, but this group also contains proteins with mobilities of gamma-2 globulin. At present we are trying to correlate the severity of the disease and the antigenic group of the patient's abnormal serum protein.

The antisera against purified multiple myeloma globulins cross-react with the proteins from other patients and with normal gamma globulin.[3] However, the homologous globulin contains antigenic groupings that are absent from all normal serum proteins or the proteins from other patients' sera. Such an anti-multiple myeloma serum, after exhaustive absorption with normal serum proteins, is completely patient-specific and cannot be used for diagnostic purposes.

These immunologic data are compatible with the hypothesis that myeloma globulins are antigenically abnormal gamma globulins; they lack some normal antigenic groupings and possess groupings that are absent from normal serum proteins.

Bence Jones protein is antigenically related to the patient's homologous abnormal serum protein and, to a lesser degree, to normal gamma globulin.[4] These urinary proteins are demonstrable in the sera of patients who excrete them. With antisera to normal gamma globulin it was shown that those patients who excrete Bence Jones proteins can be placed in two groups: A) those whose urines contain a single protein, and B) those whose urine contains two Bence Jones proteins, both of which are antigenically distinguishable from A. There is very good evidence that the antigenic difference is a function of the terminal N-amino acid composition of these proteins.[4]

Bence Jones proteins are antigenically more deficient than the corresponding serum globulins and contain some antigenic groupings of these globulins that are absent from normal gamma globulin.

Our studies of the antigenic properties of these proteins have resulted in a simple immunologic test for multiple myeloma.[5]

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DISCUSSION

PHILIP G. H. GELL (Birmingham, England): I have done some of the same sort of thing that Dr. Korngold has, some thousands of miles away, and on a very much more limited scale. Our findings are in agreement with essentially everything he has said in regard to cross-reactions, the relationships of gamma globulins, and so on. As well as using this type of technique, we have used the immunoelectrophoretic method of Grabar and Williams, which combines electrophoresis with immunologic analysis.

LEONHARD KORNGOLD: We used a method which is essentially similar: first the proteins are separated by zone electrophoresis on starch; then the starch block is cut into one centimeter strips and the proteins are eluted and concentrated. Each fraction can then be tested by the Ouchterlony technique.

PHILIP G. H. GELL: The reason I referred to that method was that it enables you to deduce things from the shape of the gamma globulin line. With normal gamma globulin, reacting with antiserum to normal gamma globulin, the line is slightly curved; with myeloma protein reacting with the same antiserum you get a much sharper curve, corresponding to a sharply defined spot of protein, and that is very characteristic.

One small point I would like to mention is about the specificity of this myeloma protein. Just before I left England we found that when very high concentrations of gamma-one globulin were present in the pathologic sera, in all sorts of different kinds of cases, such as liver disease, or disseminated lupus, such sera were apt to cross-react with an antiserum to a beta-myeloma; I did not do enough testing to verify this, but I wonder if Dr. Korngold has tested any other pathologic sera, especially of that type.

I feel in general that the use of these gel-diffusion methods has opened a very wide field of investigation, and I must say that I feel that Dr. Korngold is to be congratulated on the imaginative and critical use he has made of these techniques; for they do need to be treated very critically.

LEONHARD KORNGOLD: We have tested sera from patients with various forms of electrophoretically diffuse hyper-gammaglobulinemia: cirrhosis, lupus, endocarditis, and Boeck's sarcoid, but in these sera the gamma globulin could not be differentiated from the normal. The only proteins that cross-react with spur formation are those in the sera of patients with multiple myeloma, and this reaction seems therefore to be very specific.

We have immunized rabbits against a beta-myeloma globulin, and the antisera obtained at the first bleeding failed to react with normal gamma globulin, even though they cross-reacted with myeloma globulins of the same antigenic grouping (III) as the immunizing protein. After a second injection of this antigen, the resulting antiserum now also cross-reacted with normal gamma-globulin. The properties of your antiserum depend to a large degree on the concentration of the major and minor antigenic groupings on the proteins used for immunization, and how the rabbits react to them.

SIGMUND L. WILENS: In some cases of multiple myeloma there is considerable renal damage; in other cases there is very little. Does this make any difference in the type of globulins you find in the serum of these patients?

LEONHARD KORNGOLD: I do not know. I think that patients with multiple myeloma whose serum pattern is normal excrete Bence Jones proteins in the urine.

SIGMUND L. WILENS: You don't think that the types I, II, or III that you have distinguished are related in any way to abnormal renal function?

LEONHARD KORNGOLD: I do not know.

CHANDLER A. STETSON, Jr.: In how many patients with the diagnosis of multiple myeloma do you fail to find detectable abnormal protein in the serum?

LEONHARD KORNGOLD: Approximately 3 to 4 per cent of the sera from myeloma patients will be normal or actually have hypo-gammaglobulinemia, but they usually excrete Bence Jones protein.

CHANDLER A. STETSON, Jr.: In the mouse lines of multiple myeloma, is there Bence Jones proteinemia and is this associated with Bence Jones proteinemia, as in your patients?

LEONHARD KORNGOLD: As far as I know, Bence Jones proteins are not excreted in the urine of mice who carry the transplantable plasmacytoma tumors; at late stages of the disease proteinuria may develop, but according to Dr. Elliot Osserman it is due to the serum proteins.